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(54) Title of invention: EXTERNAL GROWTH FACTOR-NONDEPENDENT CELL LINE

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Specification

1. Title of invention

External growth factor-nondependent cell line

2. Claims

(1) An external proteinic growth factor-nondependent cell line of mammalian derivation characterized in that it self-propagates in serum-free culture medium containing no external proteinic growth factor.

(2) The cell line according to Claim 1, wherein the external proteinic growth factor comprises a combination from the group comprising insulin, transferrin, epidermal growth factor (EGF), nerve growth factor (NGF), fibroblast growth factor (FGF), ovarian growth factor (OGF), insulin-like growth factor (IGF), and somatomedin.

(3) The cell line according to Claim 1, wherein the cell line of mammalian derivation is a human-derived cell line.

(4) The cell line according to Claim 3, wherein the human-derived cell line is a human renal carcinoma-derived cell line.

(5) The cell line according to Claim 1, wherein the cell line has been transfected with SV40 enhancer gene and/or SV40 promoter gene in the chromosomes of said cell line.

(6) The cell line according to Claim 1, wherein the cell line is cell line Tr-48, which is characterized as follows:

a) Derivation: Isolated following transformation of the established human cell line TRC-29SF derived from human renal carcinoma tissue by introducing SV40 enhancer gene and/or SV40 promoter gene into the chromosomes of said cell line.

b) Morphology: Epithelial cell-like

c) Number of chromosomes: Chromosome number distribution mode characterized in showing modal number of 74 chromosomes, which is the high-triploid region.

d) Subculture: Infinite subculture

e) Functional characteristics: Human colony stimulating factor-producing

f) Cell growth performance: Grows well in serum-free medium not containing external proteinic growth factor. Doubling time 30 ± 5 hours.

g) Storage conditions: Frozen storage at -80°C to -190°C.

3. Detailed description of the invention

[Field of industrial application]

The present invention concerns an external proteinic growth factor-nondependent cell line of mammalian derivation that self-propagates in serum-free culture medium containing no external proteinic growth factor.

[Prior art and problems therewith]

Conventionally, when producing various physiologically active substances by animal cell culture, culturing is carried out using a serum medium containing bovine foetal serum or similar. However, such culture media are costly and it has also been difficult to obtain highly pure physiologically active substance products due to problems such as contamination by serum constituents. In recent years, development has thus been proceeding on serum-free media in which cells can grow without adding serum, and various serum-free media with a clear chemical composition and providing high culture reproducibility have been reported (JP 60-196186A, JP 62-107795A). Moreover, with improvements at the cell level as well, cell lines conditioned to permit prolonged subculturing in serum-free media have been reported (JP 61-289039A). However, even though these are serum-free media, during culturing it has still been necessary to add external proteinic growth factors such as insulin, transferrin, epidermal growth factor (EGF), nerve growth factor (NGF), fibroblast growth factor (FGF), ovarian growth factor (OGF), insulin-like growth factor (IGF), and somatomedin etc. when producing the various physiologically active substances, and these have been a cause of increased production costs.

[Solutions]

As a result of studying the above problem from various angles, the present inventors transformed the human cell line TRC-29SF derived from human renal carcinoma tissue by introducing SV40 enhancer gene and/or SV40 promoter gene into the chromosomes of said cell line, then isolated the cells, carried out successive subculturing and eventually succeeded in establishing a cell line not dependent on external proteinic growth factors that self-propagates in serum-free medium containing no external proteinic growth factor. This cell line was designated Tr-48 and to date has undergone about 100 subcultures in serum-free medium containing no external proteinic growth factor. There has been hardly any change in cell morphology, cell growth performance, human colony forming stimulating factor (CSF) and doubling time, and it has been recognized to be a cell line that maintains the characteristic properties of Tr-48 in a fairly stable manner. They also discovered that by culturing said cell line, it was possible to produce large quantities of CSF in a homogeneous state in serum-free medium containing no external proteinic growth factor. They discovered furthermore that because said cell line grows in inexpensive culture media containing no external proteinic growth factor, it can form host cells for gene transfection with the aim of producing all manner of physiologically active substances.

The present invention was devised on the basis of these new findings.

That is to say, the present invention is a cell line of mammalian derivation not dependent on any external proteinic growth factor, and is characterized in that it

self-propagates in serum-free medium containing no external proteinic growth factor.

The term 'external proteinic growth factor' in this invention denotes combinations of various types of external proteinic growth factor such as insulin, transferrin, epidermal growth factor (EGF), nerve growth factor (NGF), fibroblast growth factor (FGF), ovarian growth factor (OGF), insulin-like growth factor (IGF), and somatomedin etc.

To obtain the external proteinic growth factor-nondependent cell line of mammalian derivation of the present invention, established mammalian-derived cells capable of growing in serum-free medium are transformed with a vector that comprises an enhancer gene and/or promoter gene and is capable of transforming said established mammalian-derived cells and introducing the enhancer gene and/or promoter gene onto the chromosomes.# Selective culturing is then carried out in serum-free medium containing no external proteinic growth factor and the cells that grow in said medium are harvested.

Examples of established mammalian-derived cells capable of proliferating in serum-free medium include the serum-free cultured cell line AH-01 separated and conditioned from human myeloid leukaemia cells THP-1 (described in JP61-289039A) and the serum-free cell line TRC-29SF separated and conditioned from the human renal carcinoma-derived cell line TRC-29R.

Explaining this more specifically in terms of the serum-free cell line TRC-29SF separated and conditioned from

the human renal carcinoma-derived cell line TRC-29R, the human renal carcinoma-derived cell line TRC-29R developed by the present inventors (JP 61-126030A) was used as the primary cell line. The growth of this cell line is normally maintained by RPMI-1640 medium containing 5-20% of bovine foetal serum. The present inventors carried out basic research into serum-free culturing methods for human anchorage-dependent cells and developed RPMI-1640 serum-free medium containing low molecular weight gelatine (JP 60-196186). They carried out subculturing of TRC-29R using this medium. As a result, although growth performance fell markedly for 2-3 generations, they continued culturing for more than three months repeatedly changing the medium. Culturing of the cells at this time was carried out in an incubator for animal cell culture providing conditions of 5% CO_2, 95% air, relative humidity of 100%, and temperature of 37°C. Upon continuing culturing in this way, a tendency was seen for growth performance gradually to recover after 2 or 3 months, and upon subculturing for a further two months in serum-free medium containing the optimal concentrations of growth factors, a serum-free cultured cell line exhibiting almost stable growth performance was obtained, although its growth performance was slightly inferior to that of cells subcultured in serum-containing medium. They named these cells serum-free cultured cell line TRC-29SF.

Various base and modified media such as White medium, Fisher medium, Parker medium or modified versions thereof, Earle medium or modified versions thereof, Waymouth medium, Eagle medium or modified versions thereof, Pack medium, Ham medium or modified versions

thereof, Trowell medium, McCoy medium, Moore medium and modified versions thereof, Williams medium E, and also mixtures of these may be cited as media that can be used for culturing these serum-free cultured cell lines. Further illustrations include V-614 as Fisher medium of known composition, M150, M635, M703, M858, M199, CMRL-1066 and CMRL-1415 as Parker medium, NCTC109 and NCTC135 as Earle medium, MB752/1 and BME as Waymouth medium, MEM or Dulbecco's MEM as Eagle medium, N15 and N16 as Pack medium, F7, F10 and F12 as Ham medium, T8 as Trowell medium, McCoy 5A as McCoy medium, RPMI-1629, RPMI-1630, RPMI-1634 and RPMI-1640 etc. as Moore medium, and also mixtures of these. Moreover, as the medium composition of, e.g., MEM medium, 199 medium, Ham medium, RPMI-1640 medium, CMRL-1066 medium and NCTC-109 medium etc. can be adjusted by adding various amino acids, vitamins, inorganic salts or other substances such as glucose, they may be adjusted during preparation on the basis of various reports [for example 'Cell Culture Manual' p110 (May 1982) Kodansha], and for subculturing, these media needed to be spiked with an external proteinic growth factor comprising a mixture of various proteinic growth factors such as insulin, transferrin, epidermal growth factor (EGF), nerve growth factor (NGF), fibroblast growth factor (FGF), ovarian growth factor (OGF), insulin-like growth factor (IGF), and somatomedin etc.

Any enhancer gene is acceptable as long as it is an enhancer that works in cell genes of mammalian derivation, for example, papovavirus enhancer gene, immunoglobulin gene enhancer gene, LTR enhancer gene and SV40 enhancer gene, but SV40 enhancer gene is to be preferred.

Any promoter gene is acceptable as long as it is a promoter gene recognized by RNA polymerase II from cells of mammalian derivation, for example, SV40 promoter gene, rabbit β-globulin promoter gene, herpes simplex virus thymidine kinase promoter gene, human α_1-globin promoter gene, adenovirus 2 promoter gene, heat shock hsp70 promoter gene and mouse metallothionein promoter gene, but SV40 promoter gene is to be preferred.

Moreover, it is acceptable for the gene to be transfected to possess either just an enhancer gene or a promoter gene, but it is preferable to transfect with both enhancer and promoter genes. SV40 gene, which comprises enhancer and promoter genes, may be cited as an ideal example.

Plasmids and DNA-type virus bacteriophages may be cited as examples of vectors that can be used. Any plasmid is acceptable as long as it is a plasmid capable of transforming established lines of mammalian-derived cells and introducing an enhancer gene and/or promoter gene onto the chromosomes, for example, plasmid pSV2-dhfr and plasmid pSV-neo, etc. These plasmids may be used after having had the enhancer gene and/or promoter gene inserted into them using the various techniques of genetic manipulation, and in order to simplify the subsequent cell line selection, it is desirable for them to possess some kind of selection marker. Drug-resistance markers may be cited as selection markers. With DNA-type virus bacteriophages, the phage should be capable of transforming established lines of mammalian-derived cells and introducing an enhancer gene and/or promoter gene onto the chromosomes, and lambda-phages may be cited as an example.

Describing a plasmid consistent with the above objectives and the construction method for it as further illustration, this is a composite plasmid comprising pBR322-derived ampicillin-resistance gene, a replication origin gene, a neomycin resistance gene capable of being expressed in animal cells and the SV40 gene promoter and enhancer genes. It was constructed by cleaving plasmid pSV2-dhfr with restriction enzymes PvuII and HindIII, isolating a DNA comprising the 342 bp SV40 promoter-enhancer gene, and joining this DNA to plasmid pSV-neo that has been opened with restriction enzyme BamHI to construct plasmid pSV-neo-pro. The resulting plasmid pSV-neo-pro met the objectives of the present invention.

The various techniques of genetic manipulation can be employed as the method for transforming the above established mammalian-derived cells capable of growing in serum-free medium with a vector that comprises an enhancer gene and/or promoter gene and is capable of transforming the above established mammalian-derived cells capable of growing in serum-free medium and introducing an enhancer gene and/or promoter gene onto the chromosomes. For example, the electroporation, injectoscope, pricking, HVJ and liposome methods etc. may be cited as such.

To explain this in more concrete terms taking electroporation as the example, after washing the cells to be transformed, e.g. TRC-29SF, with a salt solution such as Dulbecco's phosphate buffer or Hanks solution etc., a cell suspension is obtained. After adding the target recombinant DNA, e.g. plasmid pSV2-neo-pro, to this, it is poured into a chamber with electrodes at

both ends and a high voltage pulse is applied. After standing for a set time, the cell suspension is transferred to the medium, and incubation is commenced to give transformed cells.

As mentioned above, to select transformed cells, the cell line can undergo selective culture in serum-free medium containing no external proteinic growth factor and cells growing in said medium can be harvested. On this point however, if the cells have been transformed with a vector incorporating a drug resistance marker gene, the transformed cells can be selected out more easily by carrying out primary screening in advance with culture medium spiked with the particular drug, and afterwards carrying out selective culturing in serum-free medium containing no external proteinic growth factor and harvesting the cells.

To explain selection methods for the transformed cells in more concrete terms, as primary screening, culturing should be carried out in medium spiked with a drug corresponding to the drug resistance derived from the recombinant DNA (if using plasmid pSV2-neo-pro, drugs such as neomycin or G418 etc. corresponding to neomycin resistance), and after culturing the transformed cells in ordinary medium for a set time, this is replaced with liquid medium containing the drug and culturing is continued.

Colonies of cells that have grown in the drug-spiked medium are separated and drug-resistant transformed cells are obtained. Moreover, subculturing is carried out replacing the medium used for these drug-resistant transformed cells with serum-free medium containing no

external proteinic growth factor. A cell line capable of being subcultured and showing hardly any change in growth performance in serum-free medium containing no external proteinic growth factor was separated by this procedure.

Cell lines obtained in this way were characterized in being self-propagating in serum-free medium containing no external proteinic growth factor.

For example, the above human renal carcinoma tissue-derived serum-free cultured cell line TRC-29SF underwent electroporation with plasmid pSV2-neo-pro to introduce SV40 promoter-enhancer genes onto the chromosomes, giving cells characterized in being self-propagating in serum-free medium containing no external proteinic growth factor. This cell line was characterized as follows and was named Tr-48.

a) Derivation: Isolated following transformation of human established cell line TRC-29SF derived from human renal carcinoma tissue by introducing SV40 enhancer gene and/or SV40 promoter gene into the chromosomes of said cells.

b) Morphology: Like TRC-29SF, the cultured cells grew as confluent multiangular cells on a monolayer sheet, presenting a paving stone-like appearance and showing a typical epithelial cell-like disposition. Moreover, as cell growth proceeded and a saturated state was reached, a tendency for them to grow in stacked form was seen.

c) Number of chromosomes: Chromosome distribution mode characterized in showing modal number of 74 chromosomes, which is the high-triploid region.

d) Subculture: Infinite subculture

e) Functional characteristics: Human colony stimulating factor-producing.

f) Cell growth performance: Grows well in serum-free medium containing no external proteinic growth factor. Growth curves were prepared seeding 35 mm plastic tissue culture dishes (Corning) with 2 mL of a cell suspension containing 2.5×10^4 viable cells/mL using RPMI-1640 medium adjusted to pH 7.2 by adding 7.5% sodium bicarbonate, and culturing them in an animal cell culture incubator held at 5% CO_2, 95% air, relative humidity of 100% and temperature of 37°C. The doubling time determined from the growth curve was 30 ± 5 hours.

g) Storage conditions: Frozen storage at -80°C to -190°C.

The primary cell line TRC-29R, serum-free cultured cell line TRC-29SF and cell line Tr-48 of the present invention characterized in being self-propagating in serum-free medium containing no external proteinic growth factor are compared in Table 1.

Table 1

	TRC-29R	TRC-29SF	Tr-48
Cell morphology	Epithelial cell-like	Epithelial cell-like	Epithelial cell-like
Manner of growth	Monolayer →Stacking	Monolayer →Stacking	Monolayer →Stacking
Cell growth performance	Grows in bovine foetal serum medium	Grows in serum-free medium	Grows in serum-free medium with no growth factor
Doubling time	29 ± 6 hours	35 ± 5 hours	30 ± 5 hours
Chromosome mode	74	76	74
Functional designation	h-CSF producing	h-CSF producing	h-CSF producing

Thus Tr-48 cells provide industrially advantageous conditions in terms such as growth rate and their ability to grow in serum-free medium containing no external proteinic growth factor.

As serum-free culture media with no external proteinic growth factor that can be used in the present invention, various base or modified media such as White medium, Fisher medium, Parker medium or modified versions thereof, Earle medium or modified versions thereof, Waymouth medium, Eagle medium or modified versions thereof, Pack medium, Ham medium or modified versions thereof, Trowell medium, McCoy medium, Moore medium or modified versions thereof, Williams medium E, and also mixtures of these may be cited. Further illustrations include V-614 as Fisher medium, M150, M635, M703, M858, M199, CMRL-1066 and CMRL-1415 as Parker medium, NCTC109 and NCTC135 as Earle medium, MB752/1 and BME as Waymouth medium, MEM or Dulbecco's MEM as Eagle medium, N15 and N16 as Pack medium, F7, F10 and F12 as Ham medium, T8 as Trowell medium, McCoy 5A as McCoy medium, RPMI-1629, RPMI-1630, RPMI-1634 and RPMI-1640 etc. as Moore medium, and also mixtures of these. Moreover, as the medium composition of, e.g., MEM medium, 199 medium, Ham medium, RPMI-1640 medium, CMRL-1066 medium and NCTC-109 medium etc. can be adjusted by adding various amino acids, vitamins, inorganic salts or other substances such as glucose, they may be adjusted during preparation on the basis of various reports [for example 'Cell Culture Manual' p110 (May 1982) Kodansha]. RPMI-1640 medium adjusted to pH 7.4 with sodium bicarbonate can be used as a typical example of serum-free medium containing no external proteinic growth factor.

The Tr-48 cells of the present invention are capable of being subcultured without limit in a cell culture incubator held at 5% CO_2, 95% air, and relative humidity of 100%. For comparative purposes, Fig. 1 shows the

growth curves for TRC-29SF (★ in the drawing) cultured in a cell culture incubator held at 5% CO_2, 95% air, and relative humidity of 100% at a temperature of 37°C in a serum-free medium comprising RPMI-1640 base medium spiked with 5 mg/L of insulin, 10 mg/L of EGF, 15 mg/L of transferrin, 0.1 g/L of low molecular weight gelatine, 10 mg/L of folic acid, 1 μM of ferrous sulphate, 10 mM of HEPES, 100 mg/L of penicillin, 100 mg/L of dihydrostreptomycin sulphate and 3 g/L of sodium bicarbonate, and also for Tr-48 cultured in the same medium (□ in the drawing).

CSF production capacity was confirmed as follows using methyl cellulose in accordance with the method of Niho, a colony forming method using mouse bone marrow cells ('Immunologic Experimentation Techniques', Japan Society for Immunology Ed., 1974, p927).

2.2% methylcellulose/α-MEM	1.6 mL
Equine serum	0.8 mL
Mouse bone marrow cell suspension/α-MEM	0.8 mL
Test sample or CSF reference standard	0.8 mL

The methylcellulose used was from Dow, α-MEM from '*Furo*' (Flow?) Co., the equine serum from Gibco and the CSF reference standard from Gibco (GCT-CM). As mouse bone marrow cells, mononuclear cells were isolated from the femoral bone marrow of 7-week-old ICR mice procured from Shizuoka Laboratory Animals Center, suspended in α-MEM and adjusted to 5×10^5 cells/mL.

Three 35-mm plastic dishes for tissue culture (Corning) containing the above mixture were seeded with 1 mL of the cell suspension and after culturing for 7 days in an incubator for animal cell culture held at 5% CO_2, 95% air, relative humidity of 100% and temperature of 37°C,

the number of colonies was determined regarding populations of 20 or more cells as one colony. h-CSF activity forming one colony under the above conditions was taken to be 1 unit (U).

h-CSF activity was calculated as the average of three dishes in each case.

[Advantages of the invention]

If the cell line of the present invention is used to produce various physiologically active substances, the lack of any need to add growth factors to the culture medium means that medium costs can be greatly reduced and the incorporation of impurities derived from external proteinic growth factors in the product can be avoided. The cell line of the present invention can, moreover, be used as host cells for genetic manipulation with the aim of producing a variety of physiologically active substances.

[Examples]

The invention is explained below through examples and reference examples, but is in no way limited to these.

Reference Example 1

Using the human renal carcinoma-derived cell line TRC-29R (described in JP61-126030A) as the primary cell line, cell conditioning to RPMI-1640 serum-free medium containing low-molecular-weight gelatine was carried out so as to select cell lines capable of being subcultured for prolonged periods in serum-free medium.

That is, 25 cm^3 small culture flasks containing 5 mL of RPMI-1640 serum-free medium containing low-molecular-

weight gelatine [RPMI-1640 medium containing 0.01% (w/v) of low-molecular-weight gelatine to which 5 μg/mL insulin, 25 μg/mL prostaglandin E_1 (PGE_1), and epidermal growth factor (EGF) 10 ng/mL had been added] were seeded with 1×10^5 cells/mL of human renal carcinoma-derived cells TRC-29R. These were cultured in an incubator for cell culture held at 5% CO_2, 95% air, and relative humidity of 100% and subcultured for 3 months at a subculture ratio of 1:2.5. Cell lines capable of growing in the aforesaid RPMI-1640 serum-free medium containing low-molecular-weight gelatine were obtained.

The optimal contents of the cell growth factors for these cell lines were insulin 3.1 μg/mL, PGE_1 10 μg/mL, and EGF 5 ng/mL. Further, the cells were recovered by centrifuging, washed thoroughly, recovered again by centrifuging and used to seed 25 cm^3 small culture flasks containing 5 mL of separately prepared RPMI-1640 serum-free medium containing low-molecular-weight gelatine with optimum concentrations of cell growth factors [RPMI-1640 medium containing 0.01% (w/v) of low-molecular-weight gelatine to which the cell growth factors insulin 3.1 μg/mL, PGE_1 10 μg/mL and EGF 5 ng/mL had been added]. They were cultured in an incubator for cell culture held at 5% CO_2, 95% air, and relative humidity of 100% and subculturing was carried out for 2 months at a subculture ratio of 1:2.5. A cell line exhibiting stable growth capacity in the above serum-free medium was obtained and this was named TRC-29SF.

Example 1 (Construction of pSV2-neo-pro)

Restriction enzymes PvuII and HindIII were used to cut out a 342 bp fragment in the SV40 promoter-enhancer region from plasmid pSV2-gpt made up of the *E. coli*

replication origin from pBR322, an ampicillin resistance gene site, SV40 initial promoter-enhancer region and xanthine-guanine phosphate transferase (gpt) disposed downstream of that promoter, and plasmid pSV2-neo-pro was constructed inserting this in the regular direction at the restriction enzyme BamHI site of pSV2-neo with the G418 resistance gene (neo) inserted at the same position as the previous gpt.

That is to say, 2 μg of pSV2-gpt (BRL Co.) was dissolved in 10 μL of tris-HCl buffer (pH 7.5) and reacted for 2 hours at 37°C with 6 units of PvuII (Toyobo) and 6 units of Hind III (Toyobo). This then underwent electrophoresis in 1% low-melting-point agarose gel at 150 V for one hour, and gel at the position containing a 342 bp fragment, this being the SV40 promoter-enhancer region, was cut out. This gel was dissolved at 65°C, further treated with a volume of tris-HCl buffer-saturated phenol equal to the whole, and the aqueous layer was separated by centrifuging. Adding 2 volumes of ethanol and 1/10 part of 3 M sodium acetate solution to this aqueous layer, the DNA was precipitated by centrifuging and recovered. The resulting DNA was reacted with 5 units of T4 DNA polymerase (Toyobo) for 30 minutes at 37°C in the presence of 67 mM tris-HCl buffer (pH 8.8), 6.7 mM magnesium chloride, 16.6 mM magnesium sulphate, 10 mM 2-mercaptoethanol, 6.7 mM EDTA and 33 μM $dNTP_3$ (Boehringer Mannheim) to smooth projecting ends, and give the requisite DNA fragment (this may hereafter also be referred to as the 'pro-fragment').

Likewise, 2 μg of pSV2-neo (BRL) was dissolved in 10 μL of tris-hydrochloric buffer (pH 7.5) and reacted for 2 hours at 37°C with 12 units of BamHI (Toyobo). This then underwent electrophoresis in 1% low-melting-point agarose gel at 150 V for one hour, and gel at the position comprising a 4.0 Kb linear pSV2-neo DNA was cut out. This gel was dissolved at 65°C, further treated with a volume of tris-HCl buffer-saturated phenol equal to the whole, and the aqueous layer was separated by centrifuging. Adding 2 volumes of ethanol and 1/10 part of 3 M sodium acetate solution to the aqueous layer, the DNA was precipitated by centrifuging and recovered. The resulting DNA was reacted with 5 units of T4 DNA polymerase (Toyobo) for 30 minutes at 37°C in the presence of 67 mM tris-HCl buffer (pH 8.8), 6.7 mM magnesium chloride, 16.6 mM magnesium sulphate, 10 mM 2-mercaptoethanol, 6.7 mM EDTA and 33 μM $dNTP_3$ (Boehringer Mannheim) to smooth projecting ends. The resulting DNA was reacted at 65°C for one hour in the presence of 50 mM tris-HCl buffer (pH 8.0) to dephosphorylate the 5' end, and after extracting with a volume of phenol equal to the aqueous layer, it was further treated with a volume of chloroform-phenol mixture (1:1) equal to the whole and the aqueous layer was recovered by centrifuging. Adding a 2-fold volume of ethanol to this aqueous layer, the DNA was precipitated by centrifuging and recovered to give the requisite DNA fragment (this may hereafter also be referred to as the 'linear pSV2-neo fragment').

100 ng of the linear pSV2-neo fragment obtained by the above procedure and 10 ng of the pro-fragment were reacted for 16 hours at 16°C with 10 units of T4 DNA

ligase (Tama Shuzo) in the presence of 66 mM Tris-HCl buffer (pH 7.6), 6.6 mM magnesium chloride, 10 mM dithiothreitol and 66 μM ATP.

Moreover, *Escherichia coli* DH1 (J. Mol. Biol., 557, 1983, provided by the Genetic Stock Center, Yale University Faculty of Medicine) in logarithmic growth phase cultured in 100 mL of *Buzai* (*translit*) medium comprising 0.5% Bactoyeast extract, 2% Bactotryptone (the above from Difco) and 0.5% magnesium sulphate were collected by centrifuging (10000 rpm, 2 mins) and the bacteria obtained were suspended in 40 mL of ice-cooled TfbI solution comprising 30 mM potassium acetate buffer (pH 5.8), 100 mM rubidium chloride, 10 mM calcium chloride, 50 mM magnesium chloride and 15% glycerol. After standing at 0°C for 5 minutes, they were centrifuged and the supernatant liquid discarded, whereupon they were suspended in pH 6.5 TfbII solution comprising 4 mL of 10 mM MOPS buffer (*Dotite*), 10 mM rubidium chloride, 75 mM calcium chloride and 15% glycerol. After leaving at 0°C for 15 minutes, these were taken to be competent cells.

Adding 10 μL of the aforesaid ligase-treated DNA solution to 100 μL of this E. coli cell suspension, it was left to stand at 0°C for 30 minutes. After then adding 400 μL of *Buzai* medium and leaving at 37°C for 60 minutes, the whole amount was scattered over agar medium comprising 2% Bactotryptone, 0.5% yeast extract, 0.5% salt, 1.5% Bactoagar and 50 μg/mL ampicillin and incubated overnight at 37°C to give a transformant. Twelve transformant colonies selected at random were each incubated overnight at 37°C in liquid medium comprising 2% Bactotryptone, 0.5% yeast extract, 0.5%

salt, 1.5% Bactoagar and 50 μg/mL ampicillin, after which the cells were collected by centrifuging at 12000 rpm for 30 seconds and plasmid DNA was prepared in accordance with the lysozyme-SDS method (Maniatis et al.: Molecular Cloning, pp86-94, Cold Spring Harbor 1982).

Using some of each plasmid DNA obtained, this was cleaved with PvuII and subjected to electrophoresis in 1% agarose gel. Fragments of 700, 500 and 400 bp were all found, and upon further cleaving with AatI and EcoRI and carrying out electrophoresis in 1% agarose gel, a plasmid DNA revealing a 1.2 Kb fragment was obtained. This was named pSV2-neo-pro. pSV2-neo-pro was a plasmid with SV40 promoter-enhancer incorporated in the regular direction in pSV2-neo BamHI. The above is illustrated schematically in Fig. 2 (in the drawing, A, B, E, H and P denote restriction enzyme sites, each respectively being an abbreviation as follows, A: AdaI, B: BamHI, E: EcoRI, H: HindIII, P: PvuII.; ori, □ denotes the *E. coli* replication origin, pro, ▦ denotes SV40 promoter-enhancer, gtp *(sic.)*, ▥ denotes xanthine-guanine phosphate transferase gene, neo, ▤ denotes the G418 resistance gene and Apr, ■ denotes the ampicillin resistance gene).

Example 2 (Transformation of animal cells)

Proliferating TRC-29SF cells obtained from Reference Example 1 were detached with detaching enzyme (proprietary name: Nagaze Solution, Nagase Sangyo), washed four times with phosphate buffer (PBS(-)) and adjusted to give final cell density of 2×10^4 cells/mL. The pSV2-neo-pro obtained in Example 1 was added to this

suspension to give a final concentration of 40 µg/mL. Five minutes later, it was injected into a chamber with electrodes at both ends and a high voltage pulse was applied (pulse conditions: electrode gap 3 mm, voltage 800 V, time 30 µsec; cell fusion apparatus FDH-01001 (Bioletronics of USA). After standing for 5 minutes, about 4×10^5 cells were seeded onto 60 mm diameter Petri dishes, and using RPMI 1640 medium containing 2% bovine foetal serum for the initial culture fluid, culturing was carried out for 48 hours in a cell culture incubator held at 37°C, 5% CO_2, 95% air, and relative humidity of 100%. The aforesaid RMI 1640 medium containing 2% bovine foetal serum was replaced with medium containing G-418 (200 µg/mL), and the cells were further cultured for two weeks in said incubator. Colonies that had grown in the G-418-spiked medium were separated by the filter paper method and 90 transformed cell lines were obtained.

Example 3 (Isolation of Tr-48 cell line)

The 90 lines of G-418 resistant transformants obtained in Example 2 were cultured using 35 mm diameter Petri dishes in a cell culture incubator held at 37°C, 5% CO_2, 95% air, and relative humidity of 100% using only RPMI 1640 medium (containing no external proteinic growth factor whatsoever) at a subculturing ratio of 1:2. One cell line still capable of being subcultured even after passing through 100 subculture generations was consequently obtained, and was capable of proliferative subculture in RPMI 1640 medium free of any external proteinic growth factor. Upon further continuing subculturing and examining cell morphology and nucleus form by microscopy and cell staining methods, it was

seen to be a homogeneous established line of cells. These cells were therefore named external growth factor-nondependent cell line Tr-48 and were stored frozen (Accession No. FERM P-9852).

It was established that the h-CSF production capacity of the Tr-48 obtained peaked at the time of saturation growth, reaching activity of 500-1000 units per 1 mL.

4. Brief explanation of drawings

Fig. 1 is a growth graph illustrating the culturing profile of TRC-29SF and Tr-48. Fig. 2 is a schematic representation of the construction of pSV2-neo-pro.

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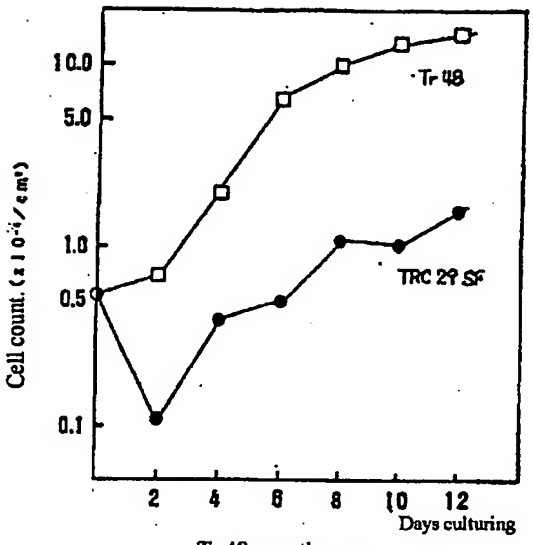
Translator's note
p5 and p9
On p5, there appears to be a grammatical ambiguity in the Japanese text. This leads, apparently, to the translation "To obtain the external proteinic growth factor-nondependent cell line of mammalian derivation of the present invention, established mammalian-derived cells capable of growing in serum-free medium are transformed with a vector that is capable of transforming said established mammalian-derived cells containing an enhancer gene and/or promoter gene, and of introducing the enhancer gene and/or promoter gene onto the chromosomes." However, this is a logical *non sequitur* and it seems more reasonable to assume that the vector both carries the enhancer gene and/or

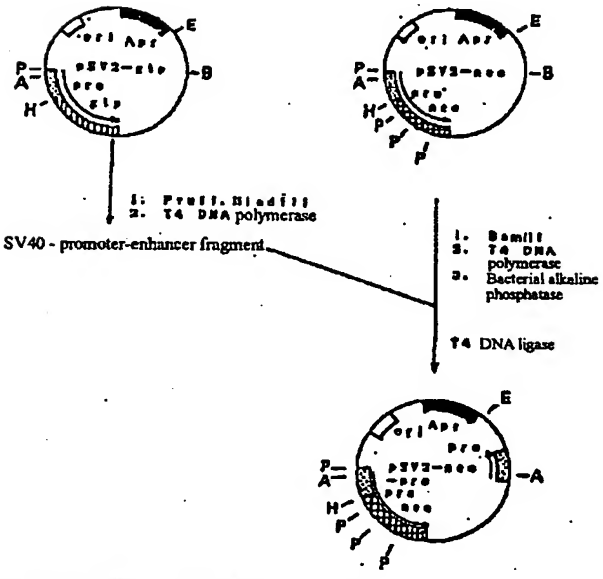
promoter gene and is capable of introducing these onto the chromosomes. This sentence has been translated taking that interpretation, though recognizing the potential for grammatical challenge. This same ambiguity is present on p9 in the paragraph beginning "The various techniques..."

Fig. 1



Tr-48 growth curve

Fig. 2



Construction of pSV2-neo-pro recombinant plasmid